

A CIRCADIAN STUDY OF LIVER ANTIOXIDANT ENZYME SYSTEMS OF FEMALE FISCHER-344 RATS SUBJECTED TO DIETARY RESTRICTION FOR SIX WEEKS.

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ABSTRACT

We examined the influences of dietary restriction (DR) on the circadian profile of liver catalase (CAT), glutathione peroxidase (GPx), and interacting systems required for removal of H₂O₂ (support systems), in 18-week old female Fischer 344 rats fed 60% of their ad libitum (AL) diet for six weeks. Food was presented to the DR animals during the early light-span. Regardless of diet, enzyme levels were generally consistent with circadian patterns. In CR animals, maximum activities often occurred at the time of food presentation. CAT and GPx activities generally were significantly higher in DR animals than in AL animals at the time of feeding. When assessing glucose-6-phosphate dehydrogenase (G6PDH) activity using saturating substrate (NADP⁺) concentrations, higher activities were seen at all times of day in the AL animals; however, when activity was measured in the presence of lower (i.e., physiologic) NADP⁺ concentrations, the reverse was true. In contrast, glutathione reductase (GR) activity was not influenced by DR.

Cytosolic levels of NADPH peaked and were higher in DR than in AL rodents prior to feeding. NADH levels were not influenced by diet, but did manifest a significant circadian pattern with a maximum occurring toward the middle of the dark span. These data suggest that even at a young age and following only a relatively brief duration of DR, there exists an enhanced enzymatic capability in rats subjected to DR to remove free radicals generated as a consequence of normal oxidative metabolism. Further, these data support emerging trends suggesting metabolic regulation of antioxidant defense systems in response to free radical generation.

INTRODUCTION

Dietary restriction (DR) is the only method proven to extend life span and retard physiologic decline as well as the onset of many age associated diseases in mammals (1-4). The mechanism by which DR induces these changes has not been delineated (5). However, it has been suggested that free radicals may be involved in both the aging process (6-12) and the development of degenerative diseases (13, 14). Consequently, it has been speculated that DR may exert its influence by either decreasing free radical formation or increasing the extent of their detoxification (5, 15-17).

Free radical generation which exceeds rate of removal may contribute to oxidative stress (18-20). Accordingly, any compromise of activities in enzymes such as catalase (CAT) and glutathione peroxidase (GPx) could lead to increased oxidative stress. These enzyme systems are dependent upon or maintained in an active state by intracellular NADPH and GSH (21). CAT utilizes NADPH to catalyze the reduction of H₂O₂, while GSH serves this function for GPx. The NADPH generating enzyme, glucose-6-phosphate dehydrogenase (G6PDH), and the GSH-generating enzyme (in the presence of NADPH), glutathione reductase (GR), play critical roles in this process (21,22).

DR has been observed to increase liver antioxidant enzyme activities in male mice and rats; however, these effects have been difficult to quantify and most have been identified in older animals (23,24). Many other biological changes which are induced by DR are detectable throughout life and since protection against free radical damage may be important at an early age, it is of interest to determine if short-term DR started early in life can significantly alter antioxidant detoxification systems when measured at various circadian stages and under specific kinetic parameters.

METHODS

Animals and Tissue Preparation

Seventy-one female Fischer 344 rats were studied. Males were not available for this study. All rats were raised at the National Center for Toxicological Research in a specific pathogen-free environment at 23°C and received the standard NIH-31 diet at 1000 CST daily. On the day animals were killed, feeding occurred at 1030 so that the animals had access to food prior to the time tissues were collected. The rats were kept on a 12 hr light (0600-1800)/dark cycle and, at 12 weeks of age, were divided into an AL control group (35 rats) and a restricted group (36 rats) which received 60% of the food consumed by the AL group. All rats were individually caged.

The rats were killed by decapitation at 18 weeks of age at 0200, 0600, 1000, 1400, 1800 and 2200 hr (n=6 at each time point for each group except for the AL group at 0600 where n=5). The liver was removed, rinsed in ice cold saline, weighed and homogenized in a buffer (3-5 volumes) designed to stabilize proteins (25). Cytosolic fractions were prepared by differential centrifugation (15), and aliquots were frozen at -70°C to await enzyme analysis. Protein concentrations were determined (26), using bovine serum albumin as a standard. Another portion of the liver was quickly frozen in liquid nitrogen and then kept at -60°C until determinations of pyridine nucleotides were made.

Catalase Assay

Catalase activity was determined spectrophotometrically by measuring the decomposition of 12.5mmol/L of H_2O_2 at 240nm (27,28). Activity was defined in traditional units as the amount of enzyme required to decompose 1 mmol H_2O_2 /min. The "effective activity of CAT" was then calculated by taking into account the period of time required for the inactivation of the enzyme by H_2O_2 (36). CAT combines with one molecule of H_2O_2 to form the obligatory intermediate known as Compound I. Another molecule of H_2O_2 is then capable of oxidizing Compound I to the inactive and oxidized Compound II. The time required for the observable inactivation of CAT at 12.5 mM H_2O_2 was defined as the time to reach a non-linear loss of absorbance versus time (when the data deviated from a linear regression by 5%). The time was determined for each reaction and multiplied by the standard activity (expressed as mmol/min/g liver) to yield the effective enzyme activity.

Glutathione Peroxidase Activity

GPx activity was determined by a modification of the automated method of Wheeler et. al., (29). GPx was measured at 30°C in a reaction mixture containing 82.2 mM phosphate buffer (pH 7.0), 0.25mM NADPH, 2.22 U GR, and 1.67 mM GSH. The reaction was started by addition of 15 mM H_2O_2 and absorbance was measured at 340 nm.

Glucose 6 Phosphate Dehydrogenase, Glutathione Reductase and Malic Enzyme Assays

The activities of GR and ME were determined using our standard analytical procedures (25). Briefly, GR activity was expressed as the amount of NADPH (0.9 mM) oxidized during reduction of GSSG (5.0 mM) in potassium phosphate buffer (100 mM, containing 2 mM EDTA, pH 7.4) by enzyme at 30° C. The change in absorbance was measured at 340 nm. For the measurement of ME activity, malic acid (20 mM) was used as a substrate in glycylglycine buffer (80 mM, containing 20 mM $MnCl_2$, pH 7.5). The reaction was started by addition of $NADP^+$ (0.76 mM) at 30°C and increase in absorbance was monitored at 340 nm. The determination of G6PDH activity was done using a previously described method (22) with our Micro Centrifugal Analyzer (Instrumentation Laboratory, Lexington, MA).

Coenzyme Quantitation

The determinations of $NADP^+$, NADH, and NADPH were modifications of the spectrophotometric assay described by Klingenberg (30). Briefly, lactate dehydrogenase (LDH) was substituted for glycerol-3-phosphate dehydrogenase in the measurement of NADH because we found the latter enzyme used NADPH as its substrate, thereby interfering with the measurement of NADPH. To determine NADH and NADPH, 2 ml of alkaline liver extract was mixed with 0.05 ml of substrate mixture (24.5mM pyruvate; 0.1 M α -ketoglutarate; 0.2M ammonium chloride, pH 7.0) in a 1 cm light-path quartz cuvette. The extinction was read for 3-4 minutes to obtain a baseline, and then for determination of NADH, 5 μ l of LDH (450 IU/ml) was added to the cuvette, mixed and extinction was read until the reaction was complete. Finally, for determination of NADPH, 5 μ l of glutamate dehydrogenase (300 IU/ml) was added and extinction was again read until no change was observed. The concentrations of NADH and NADPH were calculated by comparing the extinction of tissue extracts with standard curves made from various concentrations of NADH and NADPH.

Data Analysis

Data analysis was by ANOVA and results were reported as means (SEM). The minimum level of significance accepted was $p < 0.05$.

All data were also subjected to cosinor analysis (31) for assessing temporal changes. This method objectively determines a probability which indicates the significance of the fit of the cosine curve to the data ($P < 0.05$, the fluctuation of the variable studied is presumed to be cyclic and not random), and three rhythmic parameters and their dispersions: these are designated as the acrophase, mesor, and amplitude. The acrophase represents the crest of the fitted cosine curve. Usually, the acrophase corresponds to the time when the data values are, on the average, highest; however, it should

Table 1. Cosinor analysis of the liver cytosol antioxidant system values from AL and DR female rats.

	Feeding	PR (%)	P Value	M±SE	Amplitude	Acrophase
CAT	AL	19	0.0655	*16.4±1.9	15.1±8.4	
	DR	58	<0.0001	22.5±2.1	*19.6±3.0	10:05'±44'
GPX	AL	45	0.0001	* 5.9±0.2	1.6±.10	*03:33'±32'
	DR	52	0.0001	7.2±.04	* 1.8±.22	06:31'±34'
G6PDH	AL	44	0.0001	*90.3±2.9	*39.3±8.3	06:20'±47'
	DR	62	<0.0001	42.9±1.0	19.8±2.9	07:36'±33'
G R	AL	47	<0.0001	32.4±0.7	10.4±2.0	07:20'±44'
	DR	44	0.0001	33.8±0.8	10.5±2.2	08:30'±48'
M E	AL	53	<0.0001	*41.7±1.2	*20.0±3.5	06:37'±39'
	DR	51	<0.0001	21.7±0.7	10.7±1.9	08:14'±41'
NADPH	AL	15	0.0745	0.3±0.01	0.1±0.03	
	DR	35	0.0014	0.3±0.01	0.1±0.02	01:29'±57'
NADH	AL	27	0.008	0.2±0.01	0.1±0.02	22:06'±1:09'
	DR	47	<0.0001	0.2±0.01	0.1±0.01	21:56'±44'
Protein	AL	48	0.0001	17.8±.8	9.8±2.5	20:01'±18'
	DR	62	0.0001	17.2±.7	9.8±2.5	20:02'±18'

*Indicates significantly statistical difference

PR% = Percent rhythm and indicates the percent of time that a cosine curve is an accurate representation of a 24-hr excursion of the data.

Amplitude is the range of a 24-hr excursion of the data.

Acrophase is the cosinor calculated time of highest expected activity.

No acrophase was assigned when the cosinor was not a statistically significant fit for this data.

CAT activity is presented as effective activity ($[\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g liver}^{-1}][\text{sec}]\times 10^{-3}$).

be noted that the acrophase is not necessarily the time when the peak value was recorded. The mesor is the cosine-determined overall 24-hr mean. The amplitude is defined as one-half the total excursion best approximating the rhythm (the distance between the mesor and the crest of the cosine function). Amplitude and mesor are expressed in the original units of the variable analyzed. The minimum level of significance accepted for any of the parameters calculated by the cosine regression was also $p<0.05$.

RESULTS

Catalase Activity

When CAT activity was evaluated based on initial velocity (the usual way for expression of CAT activity using early absorbance changes versus time, when the reaction is linear), no effects of DR were detected. Additionally, there were no circadian rhythms associated with these activities; however, activities were highest just after food intake in DR but not AL animals.

Figure 1 shows effective CAT activity (effective activity = activity \times the time to deviation from linearity of absorbance versus time) as a function of time-of-day in liver from AL and DR animals. Cosinor analysis demonstrated a distinct circadian pattern of effective liver CAT activity in DR, but not in AL animals (Table 1). Additionally, the cosine function was a good predictor of the 24 hr excursion of the data ($P<0.001$), while the mesor (cosinor calculated 24 hr mean) and the amplitude were only slightly higher in DR animals. In both DR and AL animals, acrophase (cosinor calculated time of highest expected effective activity) occurred at the time of food

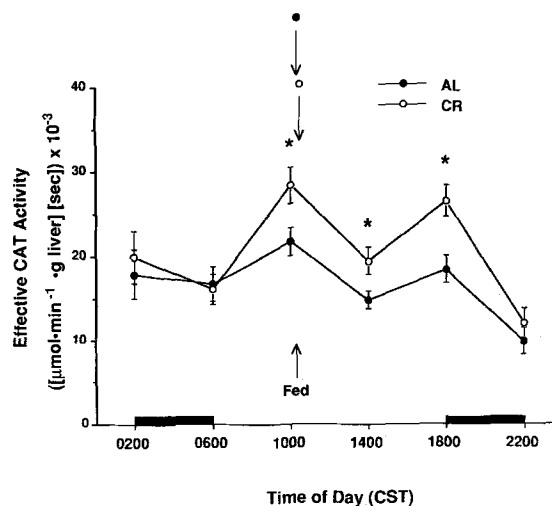


Figure 1. Effective Catalase Activity from AL and DR Rats at Different Times-of-Day. The dark bars on the x-axis indicate the times at which lights were off. DR animals were fed at 1030 hr as indicated. Arrows with symbols (○-DR and ●-AL) indicate the time-of-day for the cosinor calculated acrophase (i.e., defined reference timepoint of the crest time in the function approximating the rhythm). Error bars indicate standard deviation. * indicates time-points which are significantly different between the diet groups.

presentation. The effective CAT activity was significantly higher in DR rats as compared to AL rats at the time of food intake and for the following 8 hours while there were no statistically significant differences between the DR and AL groups at other times-of-day.

Glutathione Peroxidase Activity

GPx activity displayed a strong circadian component ($p=0.00001$) in both AL and DR animals (Table 1). The cosine function was a good predictor of temporal changes in activity as indicated by the PR% (percent rhythm refers to the percent of time that the data is approximated by an exact cosine function and values approaching 50% indicate reasonable definition of the 24 hr excursion) values (45 and 52, respectively). The 24 hr mesor was significantly increased by DR. Activities were observed to be higher in DR rats 4 hr prior to food presentation, and continued higher for the following 4 hr. This result was similar to that seen for effective CAT activity except that the peak activity was phase shifted forward by approximately 4-8 hr (acrophase = 0330 and 0631 for AL and DR rats, respectively, see Figure 2). No significant diet effect on amplitude was observed.

Glutathione Reductase Activity

GR activity was unaffected by diet. However, there was a significant circadian rhythm in GR activity from animals of either diet group (Table 1) as indicated by cosinor evaluation, and again the cosine function appeared to be a reasonable indicator of temporal change in activity. As with GPx, acrophases preceded food presentation by 1.5-2.5 hr.

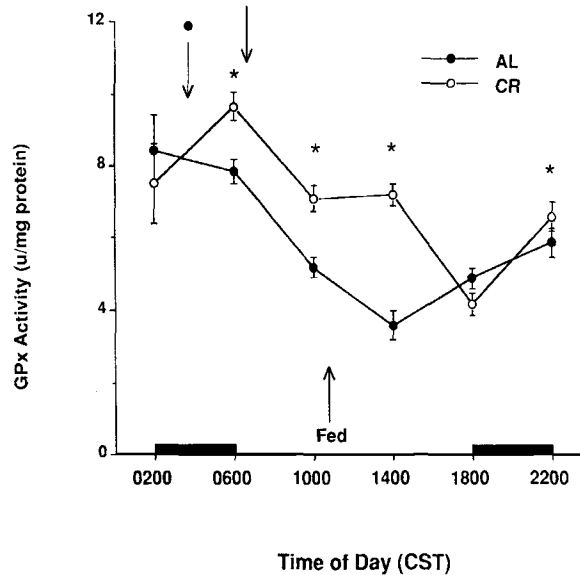


Figure 2. Glutathione Peroxidase Activity from AL and DR Rats at Different Times-of-Day. The dark bars on the x-axis indicate the times at which lights were off. DR animals were fed at 1030 hr as indicated. Arrows with symbols indicate the time-of-day for the cosinor calculated acrophase (i.e., defined reference timepoint of the crest time in the function approximating the rhythm). Error bars indicate standard deviation. * indicates time-points which are significantly different between the diet groups.

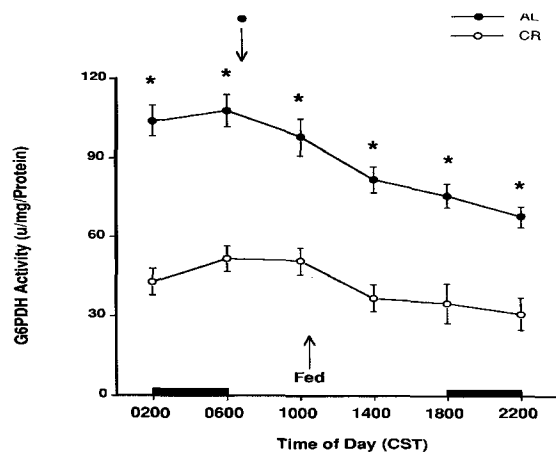


Figure 3. Glucose-6-Phosphate Dehydrogenase Activity at Saturating Substrate Concentrations from AL and DR Rats at Different Times-of-Day. The dark bars on the x-axis indicate the times at which lights were off. DR animals were fed at 1030 hr as indicated. Arrows with symbols indicate the time-of-day for the cosinor calculated acrophase (i.e., defined reference timepoint of the crest time in the function approximating the rhythm). Error bars indicate standard deviation. * indicates time-points which are significantly different between the diet groups.

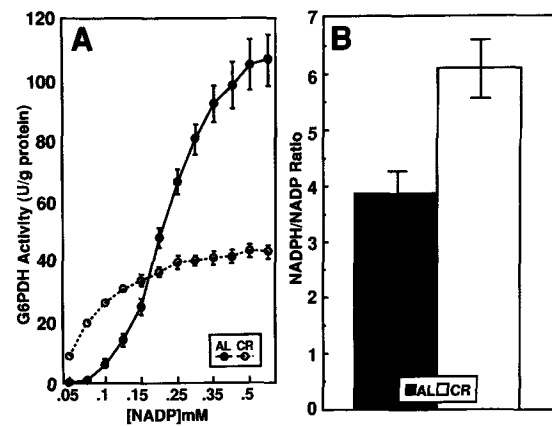


Figure 4. (Panel A) Glucose-6-Phosphate Dehydrogenase Activity versus a range of NADP⁺ Concentrations. (Panel B) The Ratio of NADPH to NADP⁺ from liver of AL and DR mice. Error bars indicate standard deviation. * indicates time-points which are significantly different between the diet groups.

Glucose-6-Phosphate Dehydrogenase Activity

Both AL and DR rats showed significant fit to the cosinor for G6PDH activity, but the cosine curve was a better predictor of the 24 hr excursion of the data in DR rats than in AL rats ($PR\%=62$ for DR and 44 for AL). Amplitude was nearly 2-fold higher in AL rats. Consistent with the results for GPx and GR, the calculated time of maximum activity was 2.5-3.5 hr prior to food presentation. G6PDH was significantly higher at all circadian stages in liver from AL rats, as was the mesor (Figure 3). However, when enzyme activity was measured across a range of NADP⁺ concentrations two different substrate saturation curves were encountered. The AL animals showed a sigmoidal curve, while the DR animals showed the characteristic Michaelis-Menten curve. This is significant because the activity was found to be higher in the liver of DR animals at physiologic levels of NADP⁺ (Figure 4). As NADP⁺ concentration increased to saturation and V_{max} was approached, the G6PDH activity seen in AL animals was significantly higher than that observed in DR rats.

NADPH Levels

Liver cytosolic concentrations of NADPH were higher 4 to 12 hr prior to feeding in samples from DR as compared to AL rats (Figure 5). However, DR did not affect the 24 hr mesor. The data from AL rats did not fit the cosine regression ($p=0.075$); therefore, no acrophase could be assigned for AL rats. The cosine regression was an accurate representation of the 24 hr excursion of NADPH levels in DR rat liver cytosol ($p=0.0014$) and acrophase was determined to be in the mid-dark (0129 hr). Maximum observed values were seen 4 hr prior to feeding and this corresponds to the acrophase for G6PDH activity. Further, at the acrophase for G6PDH activity the NADPH/NADP⁺ ratio was significantly higher in the DR group (Figure 4, panel B), although DR did not

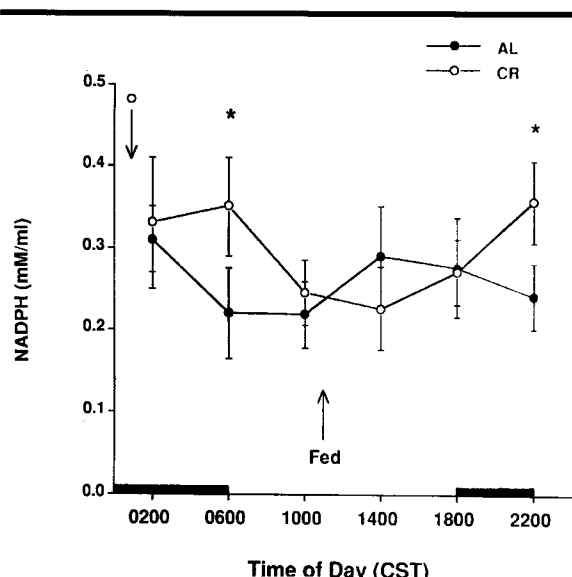


Figure 5. Nicotinamide Adenine Dinucleotide (reduced) levels from AL and DR Rats at Different Times-of-Day. The dark bars on the x-axis indicate the times at which lights were off. DR animals were fed at 1030 hr as indicated. The arrow with the open circle indicates the time-of-day for the cosinor calculated acrophase in DR mice (i.e., defined reference timepoint of the crest time in the function approximating the rhythm). The lack of an arrow with a closed circle indicates the data could not be fit to the cosinor function and no acrophase was assigned for AL rats. Error bars indicate standard deviation. * indicates time-points which are significantly different between the diet groups.

alter NADH levels. A significant fit to the cosine regression for NADH occurred for both AL and DR animals, and acrophase was in the early dark span in both groups (Table 1). The cosine function better indicated temporal activity changes in DR rats (PR% = 47 in DR and 27 in AL rats).

Cytosolic Protein

Total cytosolic protein was not affected by DR, but there was a significant circadian rhythm in both AL and DR groups, and in both cases the cosine function was a good indicator, of the rhythm as shown by the PR% values (Table 1). Acrophase occurred in early dark span in both diet groups.

DISCUSSION

Detoxification of free radicals is critical to the maintenance of macromolecular integrity, since accumulation of H_2O_2 , with potential conversion to the hydroxyl radical may lead to significant macromolecular damage. CAT and GPx are the principal enzymes which remove H_2O_2 , and each requires reducing potential in the form of NADPH generated by G6PDH. In the case of GPx, reduced glutathione is a requirement and cycled in concert with GPx in the presence of NADPH by GR (32).

Our results suggest that DR can induce circadian patterns in the activities of many of these enzymes and coenzymes. Duffy et. al., (33) found that in rats on DR,

motor activity and body temperature rise in anticipation of food presentation and, subsequently, O_2 consumption and CO_2 production increase as food is consumed. During this phase, the respiratory quotient changes in DR animals from a value of 0.82 to 1.0 indicating a switch from fat to carbohydrate metabolism. The close temporal correlation of these physiologic events with changes in the circadian rhythms of the antioxidant systems suggest that the removal of active oxygen species increases concurrently with food consumption in DR animals. By extension, free radical production may reach a maximum soon after the time of food consumption.

Interestingly, the circadian patterns observed for AL rodents were similar to those found in the DR animals where acrophases were associated with the time of food presentation to the DR rats. Acrophases for enzyme activities and coenzymes were not generally associated with the time of normal maximal food intake for nocturnal rodents (which is during the early dark span for the AL rats), but during the early to mid-light span. This suggests that presentation of food during the early light span to the DR population has a strong disruptive influence on metabolic function (and perhaps behavioral patterns) in the nocturnal AL population housed within the same animal room. Changes in physiologic parameters of DR rodents fed during the light span have been associated with interruption in quiescent activities in AL animals housed in the same animal rooms (33), since motor activity, body temperature, O_2 consumption, and CO_2 production rise appreciably as the adjacent AL animals consume significant levels of food at that time, even though primary food consumption continues to occur in the early dark period for these AL rodents. However, RQ in AL animals was not characterized by a high amplitude circadian rhythm (0.015 versus 0.076 in DR rats) as the RQ in AL animals remains nearly constant at most times, but rises appreciably in the AL animals when food is presented to DR rats (33). The lack of synchronization of rhythms for antioxidant systems to the time of normal maximal food consumption in liver of AL animals (reflective of less food consumption per unit time as compared to the DR animals) may suggest weaker coupling of antioxidant system response to oxidative stress. The lack of an increase in the antioxidant systems during the early dark in the AL animals may result in compromised protection against oxidative damage if free radical production is maximal at the time of highest food consumption. In fact, the levels of these antioxidant systems were no higher than those seen in the DR rats during the early dark phase (when metabolic rates were lowest for DR rats) (33).

NADPH did not show a circadian rhythm in AL rats, but did exhibit one in DR rats as described by cosine function. The 24 hr average (mesor) was not affected by DR; however, values were significantly higher at the sampling times prior to feeding and after feeding in the

DR rats. These higher levels of coenzyme may provide a distinct advantage for the DR animal for maintaining CAT in a reduced and active state at a time when free radical production might be expected to be high. Further, these acrophase levels of NADPH act as the substrate in the GR catalyzed reduction of glutathione which in turn, becomes available for participation in the GPx catalyzed removal of H_2O_2 . Perhaps an increased demand in AL rats for NADPH to synthesize fatty acids may contribute to their lower levels of NADPH at these times. However; similar to our previous findings (34), malic enzyme (ME) activity (an enzyme which is thought to provide NADPH for utilization by fatty acid synthetase in fatty acid synthesis) was significantly higher in AL animals (Table 1).

These diet-related changes in NADPH did not correlate with the high levels of G6PDH indicated for AL animals when the activity was measured using saturating concentrations of $NADP^+$. However, G6PDH activity measurements using physiologic $NADP^+$ concentrations (i.e., near the enzyme's K_m) revealed that activity was significantly higher in the DR rodents. Since maximal velocity would rarely, if ever, be obtained, much of the G6PDH potential in AL animals is likely never realized. It has been demonstrated that long-term increased requirements for NADPH invokes G6PDH synthesis (22). Accordingly, an increase or decrease in NADPH requirement over a sufficient period of time would be expected to yield a fluctuation in the amount of total enzyme. From these findings, it seems likely that a chronic shortage of NADPH in AL rat liver could account for the higher level of total G6PDH described herein. Also, the regulation of G6PDH in AL liver was negatively altered based on the sigmoidicity of the activity versus substrate concentration curve (Figure 4). This may be the result of altered posttranslational modifications of G6PDH which are known to occur (35). An accumulation of partially active G6PDH in AL animals along with fully active enzyme might be expected to yield the observed result.

The hypothesis that G6PDH activity is increased at physiologic levels of $NADP^+$ in DR animals is further supported by changes in the NADPH/ $NADP^+$ ratio in DR vs. AL animals. Total $NADP^+$ levels were not significantly different between AL and DR rats, but the NADPH/ $NADP^+$ ratio was significantly higher at all times-of-day in liver from the DR rats. Therefore, more of the total coenzyme pool was likely maintained in the reduced state in DR animals suggesting the possession of a larger available NADPH pool to support H_2O_2 removal. Additionally, G6PDH requires bound $NADP^+$ for stability and normal activity (22). In the AL situation, excess total hepatic G6PDH would require additional $NADP^+$. However, the absolute levels of $NADP^+$ were similar in AL and DR rat liver. Thus, there was proportionally far less cofactor for the high amounts of G6PDH in the AL liver. This situation could also account for the low G6PDH activity we have seen at physiologic concentrations of $NADP^+$ in AL rats. Likewise, the lower quantity of total

G6PDH present in the DR liver, accompanied by relatively high levels of $NADP^+$ may account for the high activity of G6PDH at physiologic levels of $NADP^+$ in DR rodents.

The present study is the first to report that CAT activity is altered by DR at young ages. Earlier, studies of CAT activity in rodents on DR did not take into account the time of feeding or the rate of inactivation of CAT. We have reported that CAT is subject to oxidation and inactivation by H_2O_2 with age, and the enzyme is resistant to this oxidation in aging animals on DR diets (36). Additionally, the levels of effective CAT activity from the liver of DR rats reported here are associated with food intake and increased metabolic activity. These observations suggest the possibility that DR either slows the rate of accumulation of compound II (21) and/or increases the rate of re-reduction of oxidized CAT (15, 37). The higher levels of NADPH and the increased NADPH/ $NADP^+$ ratio which were also found to be associated with food intake in the DR rats support the later possibility since the enzymatically catalyzed re-reduction of oxidized CAT uses NADPH as reducing substrate. GPx activity was also increased in DR animals around the time of food intake. Thus, as was the case with CAT, liver GPx activity seems to be higher in young female rats around the time of food intake and the enzyme activity is expressed in a temporal manner which optimizes H_2O_2 removal.

ABBREVIATIONS

AL: ad libitum
 DR: dietary restriction
 CAT: catalase
 CO_2 : carbon dioxide
 GSH: glutathione, reduced form
 GPx: glutathione peroxidase
 G6PDH: glucose-6-phosphate dehydrogenase
 GR: glutathione reductase
 H_2O_2 : hydrogen peroxide
 hr: hour
 LDH: lactate dehydrogenase
 ME: malic enzyme
 NADH: nicotinamide adenine dinucleotide, reduced form
 NADPH: nicotinamide adenine dinucleotide phosphate, reduced form
 $NADP^+$: nicotinamide adenine dinucleotide phosphate, oxidized form
 O_2 : oxygen
 PR%: percent rhythm
 RQ: respiratory quotient
 SOD: superoxide dismutase

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